AD________________

Award Number: DAMD17-03-1-0333

TITLE: Development of Aptamer Beacons for Antemortem Diagnosis of Chronic Wasting Disease

PRINCIPAL INVESTIGATOR: Kenneth D. Clinkenbeard, Ph.D.

CONTRACTING ORGANIZATION: Oklahoma State University
Stillwater, Oklahoma 74078

REPORT DATE: May 2004

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

BEST AVAILABLE COPY

20040917 111

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)

2. REPORT DATE
May 2004

3. REPORT TYPE AND DATES COVERED
Annual (1 May 2003 - 30 Apr 2004)

4. TITLE AND SUBTITLE
Development of Aptamer Beacons for Antemortem Diagnosis of Chronic Wasting Disease

5. FUNDING NUMBERS
DAMD17-03-1-0333

6. AUTHOR(S)
Kenneth D. Clinkenbeard, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Oklahoma State University
Stillwater, Oklahoma 74078

E-Mail: U1759aa@okstate.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)
Transmissible spongiform encephalopathies (TSE) such as mad cow disease are transmitted by ingestion of meat contaminated with infective prion protein (PrPsc). TSE of elk and deer termed chronic wasting disease (CWD) has the potential for transmission to human beings. Reliable antemortem diagnostic tests for CWD are necessary for its control. Development of a novel diagnostic probe termed aptamers to detect CWD is proposed. Once selected, the CWD aptamers will be configured as aptamer beacons that can act as molecular switches to turn "on" a novel and highly sensitive diagnostic technology termed amplifying fluorescing polymer. Objective #1 is to select aptamers for CWD PrPsc using a crossover selection strategy. Initial aptamer selection is being conducted against a tyrosyl-tyrosyl-arginine (YYR) tripeptide exposed in PrPsc but not in PrPc. YYR peptide has been constructed on cellulose, and the selection protocol optimized. Next, selection will target CWD PrPsc that we recently isolated from elk brain. We are currently developing the aptamer selection protocol for PrPsc. The final aptamer selection will crossover and negatively select against recombinant PrPc. Recombinant PrPc will be expressed from a vector construct containing the elk PrP gene obtained from Dr Katherine O'Rouke at the USDA via a materials transfer agreement.

14. SUBJECT TERMS
No Subject Terms Provided.

15. NUMBER OF PAGES
17

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

Table of Contents

Front Cover **1**

Standard Form 298 .... **2**

Table of Contents **3**

Introduction **4**

Body **4**

Key Research Accomplishments **8**

Reportable Outcomes **8**

Conclusions **8**

References **9**

Appendices **10**
Figures 10

Introduction

Transmissible spongiform encephalopathies (TSE) such as bovine spongiform encephalopathy (BSE or 'mad cow' disease) and its human equivalent variant Creutzfeldt-Jakob disease (vCJD) can be transmitted by ingestion of meat contaminated with infective prion protein (PrP^{sc}). The incubation period for TSEs is many months to years such that it is difficult to identify the source of infection and initiate effective control measures. Recently concern has been raised that a TSE of elk and deer termed chronic wasting disease (CWD) may be transmissible to human beings via ingestion of contaminated meat. Reliable antemortem diagnostic tests for TSEs are necessary for control of these diseases. Because TSEs result from mis-folding of endogenous normal prion protein (PrP^{c}) to form protease resistant PrP^{sc} isoform, diagnostic tests need to sensitively detect PrP^{sc} in samples and specifically distinguish PrP^{sc} from PrP^{c}. Monoclonal antibodies (Mab) have been produced which are both sensitive and specific for PrP^{sc}, and commercial Mab-based immunoassays are available for use in detecting PrP^{sc} in biopsy or postmortem histologic or homogenized brain tissue samples. However, a rapid antemortem tested is needed. At the time that this project was proposed, novel diagnostic probes termed aptamers had been developed to PrP^{c} but these aptamers did not distinguish between PrP^{c} and PrP^{sc} (Proske, Gilch et al. 2002). Aptamers have an advantage over Mabs because aptamers can be engineered to signal the results of a diagnostic test directly, whereas Mabs require extensive protocols to produce a detectable signal. These engineered aptamers are called aptamer beacons. We are developing aptamer beacons as molecular switches to turn "on" a novel diagnostic technology termed amplifying fluorescing polymer (AFP) when an infectious agent is present. AFP is 1,000 times more sensitive than currently available diagnostic technologies. The proposed project will develop aptamer beacons to CWD for subsequent use in developing an AFP-based antemortem diagnostic test for CWD.

Body

Aptamer selection (systematic evolution of ligands by exponential enrichment or SELEX) an iterative process of binding a pool of random sequence oligonucleotides (aptamer candidates) to the desired target, partitioning of the bound species from the unbound species, and polymerase chain reaction (PCR) copying of the bound species followed by repetition of the process until only a few oligonucleotide species that exhibit high specificity and affinity for the target remain in the pool. To overcome the negative result of Proske et al (2002) with respect to selection of aptamers that specifically recognize PrP^{sc}, we originally proposed 1) to direct aptamer selection against all linear epitopes for aa 91 to 245 of deer PrP so that pools of aptamers to all linear epitopes of PrP_{91-245} are available for subsequent crossover SELEX, and 2) to use a crossover SELEX approach (Hicke, Marion et al. 2001) to positively select for aptamers that recognize PrP^{sc} but to do recognize PrP^{c}.

Target	Selection Strategy	Advantages	Potential Pitfalls
Peptide library	Positive selection of aptamers to cover all linear epitopes of PrP_{91-245}	Yield aptamers for all linear epitopes of PrP_{91-245} so that potentially distinguishing epitopes are not missed	Aptamers to linear epitopes will not recognize epitopes peculiar to PrP^{sc}
Crude CWD PrP^{sc}	Positive selection of aptamers to all targets in crude CWD PrP^{sc} preparation	Removes aptamers from pools that do not recognize corresponding epitopes in PrPsc	Aptamers to contaminants in crude PrP^{sc} will be retained in selected pool
Purified recombinant bovine PrP^{c}	Negative selection of aptamer to PrP^{c}	Removes aptamers from pools which also recognize PrP^{c}	Negative selection will remove all remaining aptamer species

Since we proposed the strategy outlined above, two research groups have reported findings that influence our experimental approach. First, a group at Oxford selected 2'-fluoro-RNA aptamers that exhibit 10-fold higher affinity for bovine PrP^{sc} than for PrP^{c} (Rhie, Kirby et al. 2003). These researchers demonstrated the feasibility of selection of aptamers that can recognize PrP^{sc} preferentially over PrP^{c} by targeting epitopes that are more specific for PrP^{sc} than for PrP^{c} (Sayer, Cubin et al. 2004). The other group identified tyrosinyl-tyrosinyl-arginine (YYR) as the binding epitope for a monoclonal antibody that specifically recognizes PrP^{sc} from human beings, cattle, sheep, and mice (Paramithiotis, Pinard et al. 2003). These authors speculate that YYR is hidden in the interior of the normally folded PrP^{c}, but surface exposed in PrP^{sc}. Based on this finding we have altered our peptide library initial enrichment SELEX to select against immobilized YYR.

The **Statement of Work** included the only Objective 1 for Year 1:

<u>Objective 1 Select aptamers for CWD abnormal prion (PrP^{sc})</u> (Months 1-15)

Tasks 1.1 Develop protocol for peptide library SELEX (Months 1-3)

1.2 Conduct SELEX with peptide library array for deer prion (PrP_{91-245}) (Months 3-9)

1.3 Prepare and assess preparation of crude cervid PrP^{sc} (Months 4-9)

1.4 Conduct SELEX for crude cervid PrP^{sc} (Months 9-12)

1.4 Conduct negative SELEX for bovine recombinant normal prion ($rPrP^{c}$) (Months 12-15)

1.5 Assess enrichment of aptamer pool for target binding following round 6, 9, and 12 of SELEX (Months 9, 12, and 15)

Deliverable: Reduced aptamer pools selected for sensitive and specific recognition of PrP^{sc}

Progress on Objectives

1.1 Develop protocol for peptide library SELEX (Months 1-3)

The SPOTs kit from Sigma Genosys was used to custom construct YYR peptides immobilized on a cellulose membrane. As mentioned above, in July 2003, a differential epitope was described which enabled antibodies generated against this epitope to discriminate the two forms of PrP (Paramithiotis, Pinard et al. 2003). This epitope is a tandem YYR motif located at residues 152-154 and 165-167 on the elk prion protein. We will use this peptide to direct the aptamer pool toward recognition of this motif on the abnormal protein. We believe this peptide will provide an excellent target for aptamer binding because of the positive charge on the arginine and opportunities for hydrogen bonding on the tyrosine residues. The use of SPOTs rather than purchase of custom peptides from commercial sources will allow us to optimize our SELEX protocol with a proven differential target while maintaining the ability to rapidly construct any sequence of peptides that future experiments may dictate. We have successfully constructed a GYYR peptide (glycine added at N-terminal for spacing) and verified this with quantitative amino acid analysis by an outside laboratory.

1.2 Conduct SELEX with peptide library array for deer prion (PrP91-245) (Months 3-9)

Our aptamer pool is comprised of a random thirty nucleotide region flanked by twenty-four nucleotide primer binding sites (Figure 1). The reverse primer was biotinylated on the 5' end to allow for removal of negative strands. This construct forms the seventy-eight nucleotide template for PCR amplification. Initial optimization experiments were performed to amplify this oligonucleotide with high fidelity to high copy number. Baseline experiments were completed to identify likely polymerase enzymes which would function well in our application, and then these were used as a basis for 'fine tuning' the amplification.

SELEX is an iterative process that involves interaction of an oligonucleotide pool with a target, separation of binding and non-binding species, and amplification of binding species which then forms the pool for subsequent selection (see Figure 2). Since the amplified product serves as the pool from which the next set of binding ligands is selected, it is imperative to be able to amplify very low copy numbers of oligonucleotides with high fidelity. Amplification of short oligonucleotides such as this 78mer presents unique challenges. Primer dimer formation is very problematic when trying to amplify very low copy numbers of recovered, binding aptamers. This has been overcome by designing our primer binding sites whose complements have no capacity for dimerization. Another challenge is the development of high molecular weight parasites during amplification. This problem seems to occur when the reaction is out of balance as illustrated by Figure 3. Optimized cycling parameters and reactant concentrations are given in Figure 4. Figure 5 shows the optimized product on a 4.5 % agarose gel stained with Sybr Gold.

Cellulose as an immobilization strategy for SELEX has not been used before. It was therefore necessary to study the interaction of an oligonucleotide with cellulose. This was done using a ^{33}P radiolabeled aliquot of our oligonucleotide pool, and the resultant calculated interaction was less than 5% (see Figure 6). Initial attempts at SELEX with peptides immobilized on cellulose are still ongoing and being optimized.

1.3 Prepare and assess preparation of crude cervid PrP^{sc} (Months 4-9)

In December 2003, Dr. Blair traveled to Washington State University to the laboratory of Dr. Katherine O'Rourke. This laboratory routinely purifies PrP^{sc} from deer, elk, and sheep samples. Over the course of three days, Dr. Blair was able to observe and perform this purification. Briefly, samples were homogenized in lysis buffer, treated with proteinase K, and precipitated with phosphotungstic acid. PrP monoclonal antibody F99/97.6.1 was used to demonstrate PrP^{sc} on western blots of SDS PAGE preparations of the product with chemiluminescent imaging (Figure 7). We have successfully enriched crude brain homogenates of known infected elk for PrP^{sc} using the methods of Wadsworth et al as modified by the O'Rourke laboratory (Wadsworth, Joiner et al. 2001) utilizing alkaline phosophatase imaging at Oklahoma State and are in the process of optimizing it for target presentation (Figure 8). Prior to working with PrP^{sc} in our laboratory, it was necessary to obtain a permit from USDA. This has been accomplished (USDA permit # 50959).

1.4 Conduct SELEX for crude cervid PrP^{sc} (Months 9-12)

SELEX conditions are currently being optimized for presentation of the crude target. Additional enrichment steps (such as dialysis and/or ultracentrifugation) may be necessary to isolate PrP^{sc} to near purity. We are also considering the use of capillary electrophoresis as a presentation strategy for this target (Mendonsa and Bowser 2004). Capillary electrophoresis has been shown to have significant advantages over other methods. The primary advantage is the much greater ability to partition free (non-binding) oligonucleotides from those bound to a target in solution. This high degree of partitioning allows selection of binding aptamers within a very few rounds of SELEX. In addition, the lack of an immobilization substrate obviates the need for a negative selection step to such substrate. The primary preliminary work necessary to do this will be measurement of the isoelectric point (PI) of cervid PrP^{sc} because previously calculated and measured PI's have been highly variable (Sklaviadis, Manuelidis et al. 1986; Schmerr, Cutlip et al. 1998). This recent development in aptamer selection may hold great promise for improving the efficiency of SELEX protocols, especially for difficult targets such as PrP^{sc}.

1.5 Assess enrichment of aptamer pool for target binding following round 6, 9, and 12 of SELEX (Months 9, 12, and 15)

Enrichment of aptamer pool will be done through radiolabeled binding studies and further characterized through cloning and sequencing of selected aptamers.

Key Research Accomplishments

- Protocols for SELEX using cellulose immobilized peptides developed
- Immobilized peptide YYR made in preparation for SELEX
- Crude elk CWD prion protein target prepared and assessment are currently underway
- SELEX protocol for use with crude elk CWD prion protein under development
- Materials transfer agreement with USDA arranged to obtain recombinant elk normal prion protein genetic construct

Reportable Outcomes

Presentations:
Clinkenbeard KD, Jean Clarke J, Malayer JR, Hancock LF, Moon JH, Guo N, Timothy A. Snider TA, Dye R, Wang S. Aptamers for Detection of Biowarfare Agents. *Army Research Office Workshop for on the Chip Detection of Biological and Chemical Molecules.* 2004, Raleigh, NC.

Blair JL, Selection of an Aptamer to the Misfolded Prion Protein of Chronic Wasting Disease Oklahoma State University Graduate College Research Symposium (03-05-2004)

Blair JL, Sims W, O'Rourke KI, Clinkenbeard KD, Discovery Research to Select an Aptamer to the Mis-folded Prion Protein of Chronic Wasting Disease. Oklahoma State University CVM Phi Zeta Research Day (03-18-2004)

Blair JL, Clinkenbeard K, and O'Rourke K. Selection of an aptamer to the misfolded prion protein of chronic wasting disease. Oklahoma State University Food and Agricultural Products Research and Technology Center Symposium (04-19-2004)

<u>Graduate Student(s) Supported</u>: Jeffrey Blair, DVM enrolled in Veterinary Biomedical Sciences PhD program
<u>Funding Applied for Based on this Work Supported by this Award</u>: Small Business Technology Transfer (STTR) Program, Proposal Number: A045-027-0237; Topic Number: A04-T027; Ruminant B-Lymphocyte Yellow Fluorescent Protein Aggregation Bioassay for Elk Chronic Wasting Disease

Conclusions

We have completed much of the preliminary work necessary to begin selection of an aptamer which will specifically recognize cervid PrP^{sc} and distinguish it from PrP^{c}. The groundwork we have laid with our PCR optimization, production and purification of

SELEX targets, immobilization strategies, and radiolabeling experiments will be used throughout this project as we move forward in aptamer selection. Once an aptamer is selected, it will be engineered as a signaling moiety for the rapid antemortem diagnosis of CWD. In addition, the strategies used in selection of this aptamer can be rapidly adapted to selection of aptamers for other TSE targets, such as BSE and CJD such that, once optimized, our strategy may result in an important step forward in the diagnosis of all of these important diseases.

We have also established important collaborative relationships with other prion disease research laboratories around the United States, including Katherine O'Rourke's laboratory at Washington State University, Bruce Chesebro's group at the National Institutes of Health Rocky Mountain Laboratory, Dr. Elizabeth Williams at the University of Wyoming and the Wyoming State Veterinary Laboratory, and Dr. Mike Miller at Colorado State University. Dr. Blair has visited each of these individuals with the purpose of establishing relationships and soliciting input on this project. This effort has been very fruitful so far and will lay the groundwork for future collaborative investigations between our laboratories.

References

Hicke, B. J., C. Marion, et al. (2001). "Tenascin-C aptamers are generated using tumor cells and purified protein." J Biol Chem **276**(52): 48644-54.

Mendonsa, S. D. and M. T. Bowser (2004). "In Vitro Evolution of Functional DNA Using Capillary Electrophoresis." JACS **126**(1): 20-21.

Paramithiotis, E., M. Pinard, et al. (2003). "A prion protein epitope selective for the pathologically misfolded conformation." Nat Med **9**(7): 893-9.

Proske, D., S. Gilch, et al. (2002). "Prion-protein-specific aptamer reduces PrPSc formation." Chembiochem **3**(8): 717-25.

Rhie, A., L. Kirby, et al. (2003). "Characterization of 2'-fluoro-RNA aptamers that bind preferentially to disease-associated conformations of prion protein and inhibit conversion." J Biol Chem **278**(41): 39697-705.

Sayer, N. M., M. Cubin, et al. (2004). "Structural determinants of conformationally selective, prion-binding aptamers." J Biol Chem **279**(13): 13102-9.

Schmerr, M. J., R. C. Cutlip, et al. (1998). "Capillary isoelectric focusing of the scrapie prion protein." J Chromatogr A **802**(1): 135-41.

Sklaviadis, T., L. Manuelidis, et al. (1986). "Characterization of major peptides in Creutzfeldt-Jakob disease and scrapie." Proc Natl Acad Sci U S A **83**(16): 6146-50.

Wadsworth, J. D., S. Joiner, et al. (2001). "Tissue distribution of protease resistant prion protein in variant Creutzfeldt-Jakob disease using a highly sensitive immunoblotting assay." Lancet **358**(9277): 171-80.

Appendices

Figures

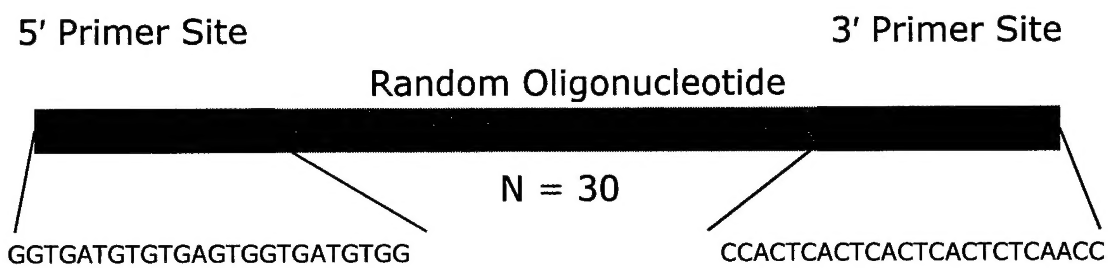

Figure 1 Depiction of aptamer construct. Random central 30mer depicted in grey.

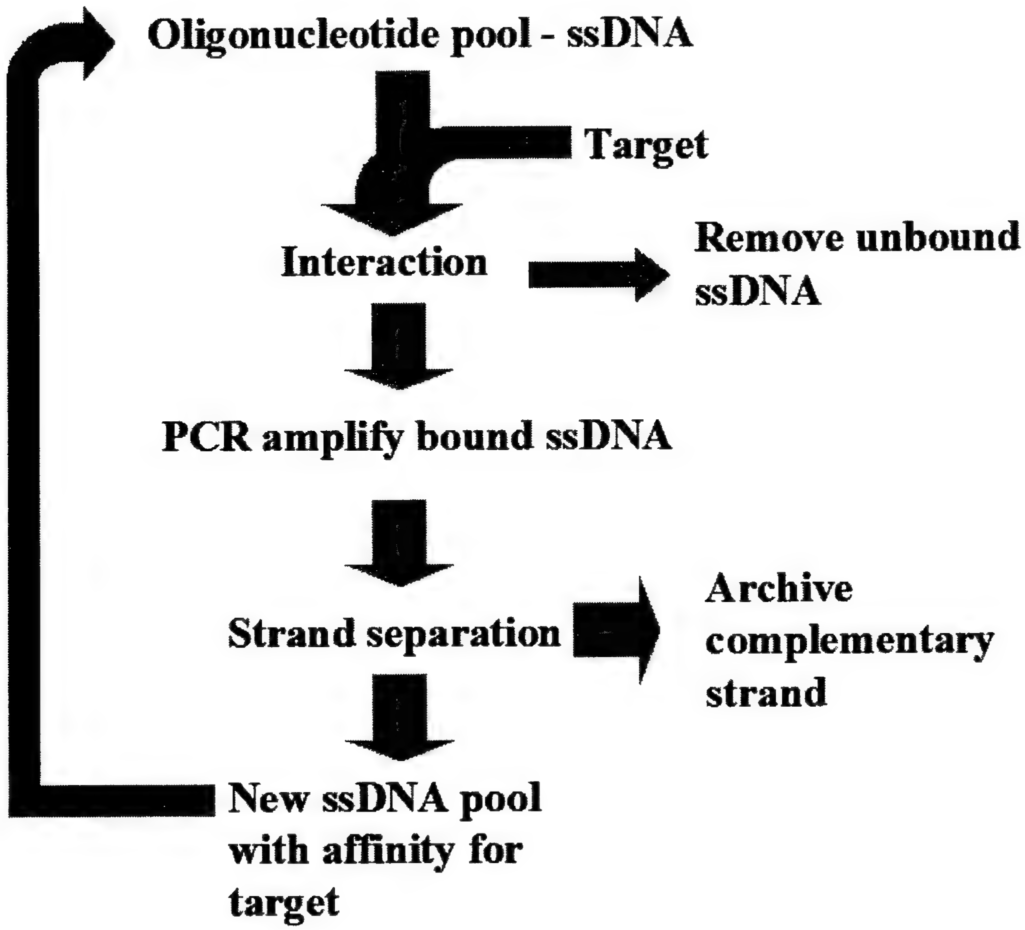

Figure 2 Illustration of SELEX

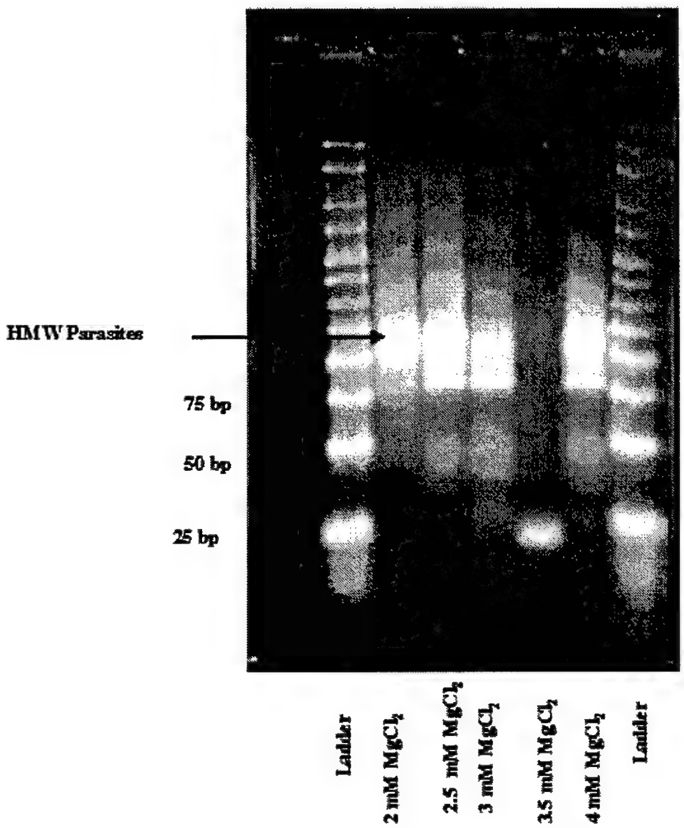

Figure 3 Illustration of high molecular weight (HMW) parasite development in PCR with high magnesium concentrations.

Reaction Mix:
Immobuffer (16 mM (NH4)2SO4, 0.1 M Tris-HCl, 0.01%Tween-20)
0.5mM each dNTP
1mM $MgCl_2$
1uM Primer 1
1 uM Primer 2
1 U Immolase

PCR Cycles:
7 min @ 95° C
X 29
30 sec @ 55° C
30 sec @ 72° C
15 sec @ 94° C
10 min @ 72° C

Figure 4 Optimized reaction mix and cycling parameters for PCR amplification of aptamer pool

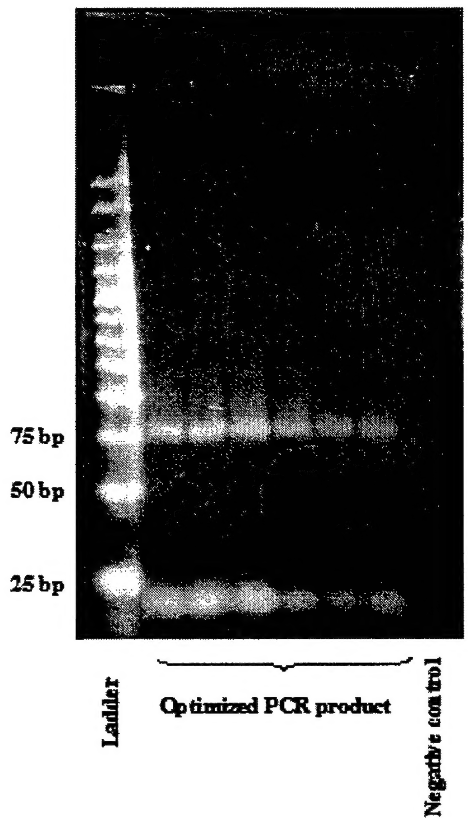

Figure 5 Individual, optimized PCR reaction product on 4.5% Agarose gel

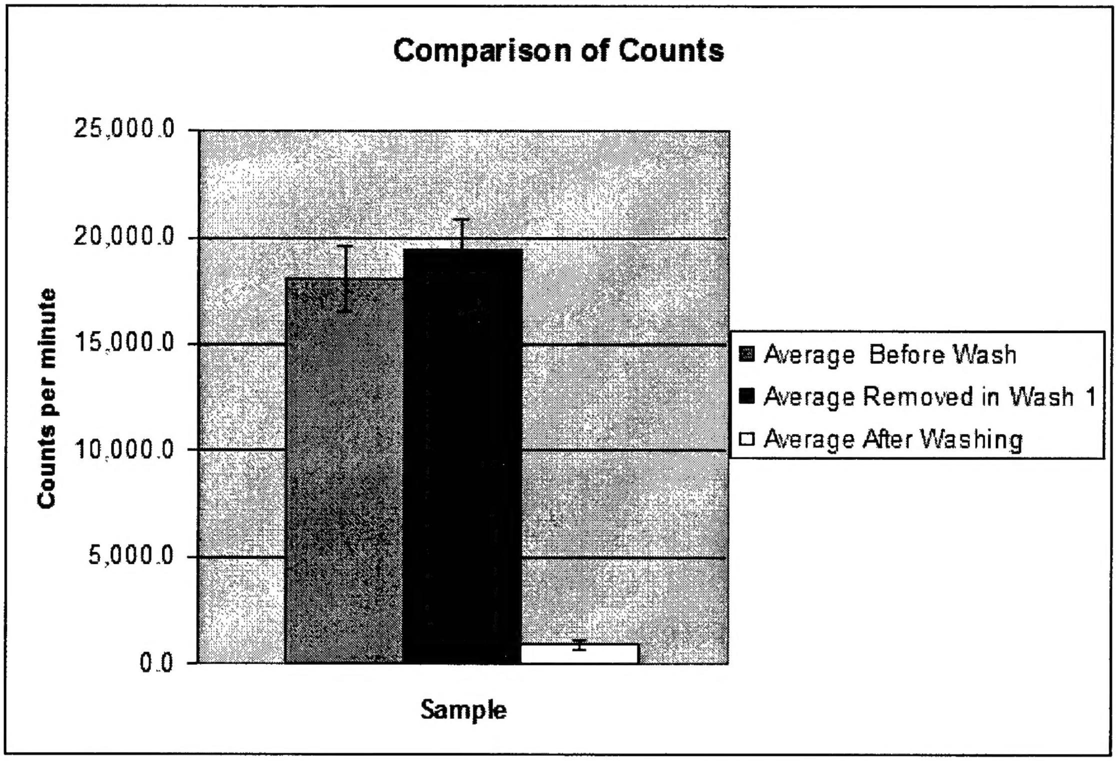

Figure 6 Interaction of radiolabeled oligonucleotide pool with cellulose membrane

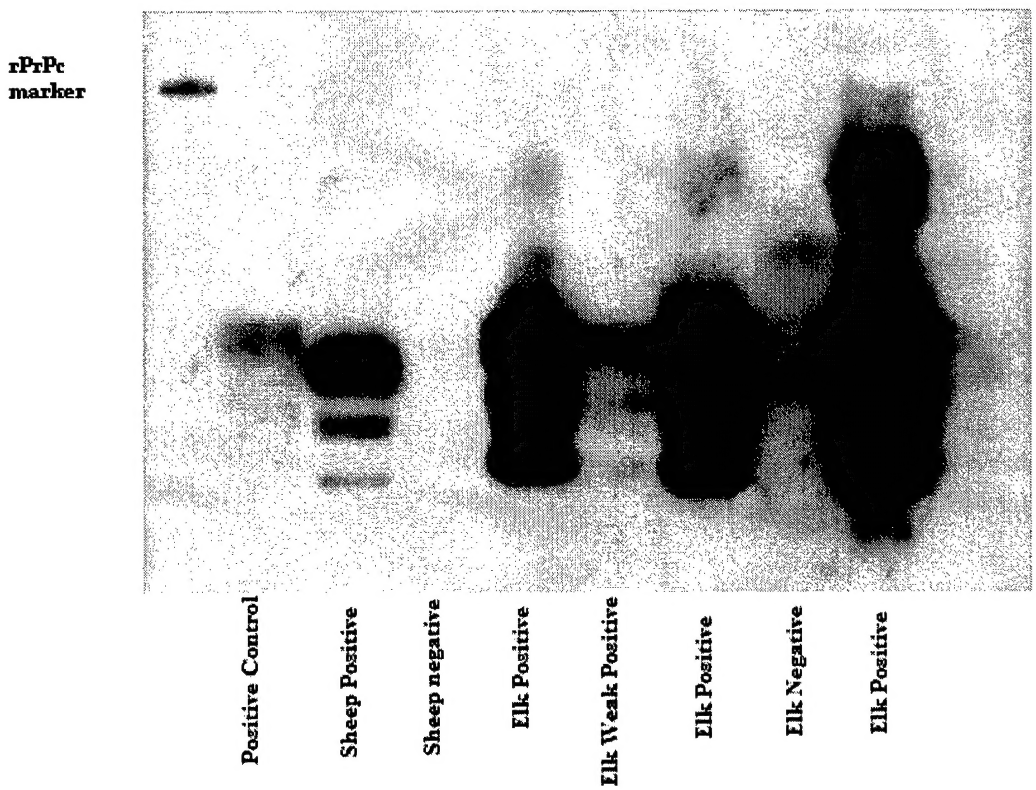

Figure 7 Western blot of PrP^{sc} from known positive elk and sheep samples performed at O'Rourke Laboratory

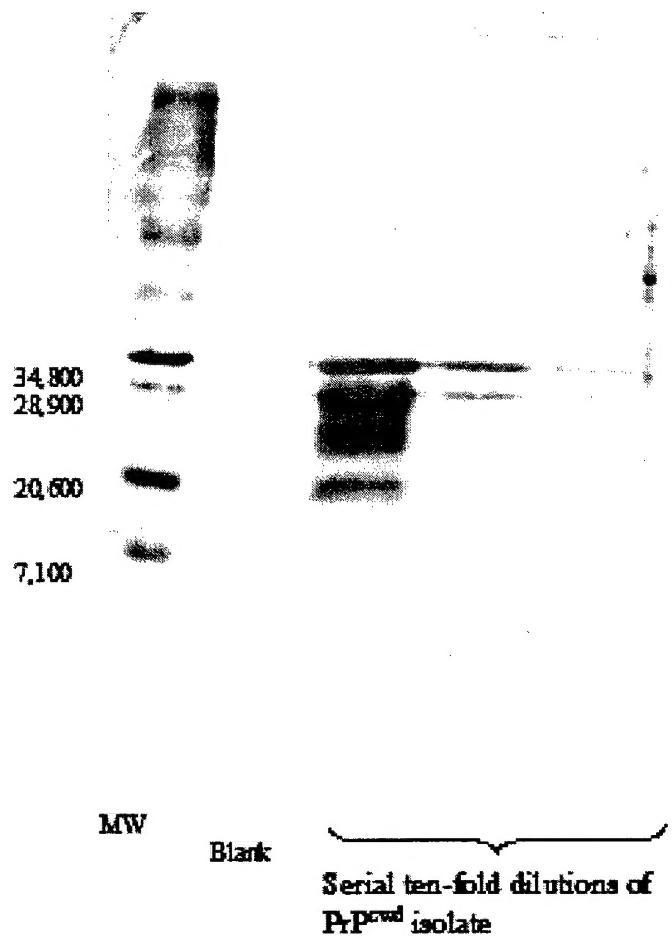

Figure 8 Western blot of PrP^{cwd} (PrP^{sc}) isolate performed at OSU